Bloco 3: Aplicações das tecnologias emergentes na reprodução animal

Session 3: Application of emerging technologies on animal reproduction
Importance of micro RNAs in developmental biology with respect to gametes and embryos

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ABSTRACT

Background: Advances in the analyses of human and other higher eukaryotic genomes have disclosed a large fraction of the genetic material (ca 98%) which does not code for proteins. Major portion of this non-coding genome is in fact transcribed into an enormous repertoire of functional RNA molecules (ncRNAs) rather than encoding any proteins. Broadly ncRNAs fall into three size classes namely, ~20 nucleotides for the large family of microRNAs, to 25-200 nucleotides for other different families of small RNAs and finally to over thousands of nucleotides for macro ncRNAs involved in eukaryotic gene regulation. Among the ncRNAs that have been revolutionized our understanding of eukaryotic gene expression, microRNAs (miRNAs) have recently been emphasized extensively with enormous potential for playing their pivotal roles in diseases, fertility and development. The miRNAs are estimated to comprise 1–5% of animal genes or a given genome could encode nearly thousands of miRNAs. Moreover, a typical miRNA regulates hundreds of target genes and altogether they could target a large proportion of genes up to 30% of the genome.

Review: It was reviewed the involvement of miRNAs for reproductive biology in mammals known so far. Several studies expanding from identification and expression profiling to functional involvement of miRNAs in the ovary have been carried out in different animal species. Several studies highlighted the expression and regulation of some individual miRNAs in different ovarian cells especially in oocyte and granulosa cells. Further more the impact of miRNAs for embryonic development was considered. The well-orchestrated expression of genes that are derived from the maternal and/or embryonic genome is required for the onset and maintenance of distinct morphological changes during the embryonic development. Optimum regulation of genes or critical gene regulatory event in favor of early embryonic development have been shown directly (individual miRNAs study) or indirectly (disrupting miRNAs biogenesis) under the control of miRNAs. Finally, miRNA effects on DNA methylation pattern are reported by the review. Reversible DNA methylation and histone modifications are known to have profound effects on controlling gene expression. Correct DNA methylation patterns are paramount for the generation of functional gametes with pluripotency states, embryo development, placental function and the maintenance of genome architecture and expression in somatic cells. Aberrancies in both the epigenetic and in the miRNA regulation of genes have been documented to be important in diseases and early development. Interestingly, it has been evident that there is an effect of miRNAs on epigenetic machinery. On the other hand miRNA expression also found to be controlled by epigenetic mechanisms.

Conclusion: Significant advancements have been made in recent years on understanding the involvement of miRNA’s in ovarien function, gene regulation well as early embryonic development. Since this area of research is rapidly moving forward it is expected that a lot of information regarding miRNA-mediated posttranscriptional gene regulation and their epigenetic regulation in ruminant reproduction biology will be known within the next several years. Studies to identify the specific miRNAs, their target genes and post transcriptional regulatory network will further shed light on the importance of specific miRNA both for the development and function of reproductive tissues as well as disease condition. Once relevant miRNAs and functional targets are identified, possible clinical use for these molecules will represent the next front line and may lead to novel strategies for better enhancing or manipulating reproductive efficiency.

Keywords: miRNA, siRNA, RNA interference.

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I. INTRODUCTION

Proteomic analysis of genome sequences in the past, highlighted only mRNA-coding genes and non-protein-coding transcripts were often overlooked. Genomic analysis in the last decade however evident that with an increase in genome complexity, the protein coding fractions of genome is much fewer compared to non-coding portion. It is estimated, that around 98% of the transcriptional outputs of eukaryotic genomes consist of large proportion of RNAs, those do not encode proteins [1]. This vast untranslated fraction of the genome harbors thousands of genes which lead to transcription of a remarkable number of functional non-coding RNAs [64]. Beside the initial discovery of the ribosomal RNAs, small nuclear RNAs and transfer RNAs that are involved in mRNA splicing and translation, many more classes and types of recently discovered ncRNAs are now also known to be involved in the regulatory functions namely, but not limited to, transcriptional and post transcriptional gene regulation, chromosome replication, RNA processing, site-specific RNA modification, DNA methylation, telomere synthesis and length differentiation, protein degradation and protein translocation [35,91]. Ongoing identification of new classes of non-coding RNAs (ncRNAs) and new member of existing classes presently underscores the paramount importance of ncRNAs function at many levels essential for gene expression and genome stability.

Even though the types of ncRNAs are emerging from occasional discoveries with varying in size (nt), mechanisms of biosynthesis and their regulatory mechanisms but their list is continuously and tremendously increasing and getting appreciation for their functional importance. Broadly, ncRNAs could be differentiated into three classes according to their range in size. Among them, the tiny one ranges in size about 20 nucleotides (nt) for a large family of miRNAs that have been found to modulate development of mammals and engaged in diseases development as well as contributing to the fertility of different species through post transcriptional gene regulation. The group of ncRNAs ranging in size 100-200 nt are designated as small RNAs commonly found as translational regulators in bacteria and some other species as well. Lastly, the ncRNAs comprised of majority of the longer transcripts to over 10000 nt in size are called as macro ncRNAs involved in epigenetic regulation of gene expression in eukaryotes [40,91]. In contrast to the uncertainty surrounding the function of most mammalian macro ncRNAs, imprinted macro ncRNAs have clearly been identified as regulator of flanking genes by DNA methylation [47]. Small non coding RNAs such as miRNAs, short interfering RNAs, piwi-interacting RNAs and short nucleolar RNAs are associated with trans-acting functions, where as macro ncRNAs are so far only associated to cis-acting functions.
However, knowledge on the types and the members of each type are still limited to most of these biochemically abundant species of ncRNAs and many of them yet to be discovered. It is likely that there are many more ncRNAs than was ever suspected. Here we review the recent reports on the small non-coding RNAs with particular emphasis on miRNAs in details and some other selected small ncRNAs briefly in animal models focusing on their diverse roles in the physiology of reproductive cells (germ cells) and tissues (testis, ovary, endometrium, oviduct and embryo) together with their implications for ruminant reproductive biology.

II. SMALL NON-CODING RNAS AND THEIR MECHANISMS OF GENE REGULATION

The notion of the sncRNAs is not new - for example 5S rRNAs, U6 RNA, snoRNAs, BC200 RNA, etc. were discovered long before, but it is only recently been highlighted because of growing list of classes and members of every class of different other sncRNAs which are found to be physiologically important as riboregulator. MiRNAs are the well characterized ones getting more attention to the scientific community due to their high level of importance. Diverse expression pattern of miRNAs and high number of their potential target mRNAs suggests their involvement in the regulation of various developmentally related genes at post-transcriptional level [4,6,11,21,50,51,78]. The tiny (18-24 nt in length) and single-stranded, derived from primary transcripts termed as “pri-miRNAs”, having an RNA hairpin structure of 60-120 nt with a mature miRNA in one of the two strands (Figure 1). This hairpin in turn is cleaved from the pri-miRNA in the nucleus by the double-strand-specific ribonuclease, Drosha [54]. The resulting precursor miRNA (pre-miRNA) is transported to the cytoplasm via a process that involves Exportin-5 [114] and subsequently cleaved by Dicer [53] to generate a short, double-stranded RNA duplex. One of the strands of the miRNA duplex is incorporated into a protein complex termed RNA induced silencing complex (RISC). RISC is guided by the incorporated miRNA strand to mRNAs containing complementary sequences in 3´ untranslated region to 7- to 8-nt region of 5´ end of miRNA called seed sequence, which primarily results in inhibition of mRNA translation [77] (Figure 1). Blocking the translation of mRNAs occurs through interaction of RISC with eukaryotic translation initiation factor 6, which prevents assembly of 80S ribosomes [22], or through inhibition of translation after initiation [42]. Recent reports have also indicated that miRNA, with or without perfect sequence complementarity, can cause an increase in mRNA degradation by endonucleolytic cleavage or deadenylation, respectively [42] or changes in proteins associated with RISC can cause a shift from translational inhibition to translational enhancement [72,98]. Those mRNAs which are repressed by miRNAs are further stored in the cytoplasmic foci called P-bodies [58,59,80]. MicroRNAs have found to play an integral part of animal gene regulatory networks as one of the most abundant classes of gene regulators. Several studies have shown the involvement of miRNAs in animal development and diseases.

Despite the fact that animal miRNAs, which are the focus of this review, hence a significant importance in the reproductive process, the other types of small noncoding RNA with distinct properties also deserve more attention. Small interfering RNAs (siRNAs) differ from miRNAs mainly in their Origin. They are the products of long, Dicer-processed, double-stranded (ds) RNAs that silence genes by cleaving their target mRNAs (Figure 2) [reviewed in 24]. The RNAi was first discovered by introduction of long ds RNAs into C. elegans [28]. Like endogenous miRNAs, long dsRNAs are processed by the Dicer-TRBP-PACT complex [reviewed in 24,79]. This dsRNA-processing step creates RNA with 2-nt overhangs at their 3´ ends and phosphate groups at their 5´ termini. The anti-sense strand of siRNA, known as the guide strand serves as the template for sequence-specific gene silencing by the RNAi machinery (Figure 2). The sense strand is known as the passenger strand. Subsequent to Dicer processing, the 21–23 nt guide strand of duplex siRNA is loaded into Ago2 to form the effector siRISC. Ago2 is the endonuclease responsible for the cleavage activity of siRISC. With perfect base pairing and formation of an A-form helix structure between the siRNA guide strand and its target mRNA, siRISC cleaves its target 10–11 nt from the 5´ end of the guide siRNA strand, and the complex is recycled for the next round of target mRNA cleavage. mRNAs cleaved by siRISC are subsequently degraded by cellular exonucleases, resulting in robust depletion of target genes [reviewed in 24,79].
III. FUNCTION OF miRNAs WITH RESPECT TO REPRODUCTION

The miRNAs are estimated to comprise 1–5% of animal genes [11,13,14] or a given genome could encode nearly thousands of miRNAs [13]. Moreover, a typical miRNA regulates hundreds of target genes [17,49,55,110] and altogether they could target a large proportion of genes up to 30% of the genome [56]. Changes in the expression of even a single miRNA found to have a significant impact on the outcome of diverse cellular activities. Inhibition of miRNA biogenesis has been found to result in developmental arrest in mouse and fish [15,31,107] and female infertility in mouse [73,74]. Investigation on the potential role of miRNA in reproduction up-to-date has been accomplished by the different approach. First, by identifying the population of miRNAs in the germ cells and reproductive tissues through cloning method. Second, by investigating the expression of candidate miRNA or group of miRNAs using microarray platform or RT-PCR approach. Third, by localizing candidate miRNA in the tissue or cell using in-situ hybridization approach. Forth, by knocking down global miRNA expression by creating Dicer1 knockout mice. Finally, by investigating specific miRNA function through using the oligonucleotide inhibitors and/or miRNA mimics or precursors. Accounting the studies and approaches published so far, the following sub-sections describe the role of miRNAs with respect to reproductive biology.

IV. EXPRESSION AND REGULATION OF miRNAs IN THE MAMMALIAN OVARIAN CELLS AND THEIR FUNCTION

Dynamically regulated, complex and coordinated ovarian functions include sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis are under control of closely coordinated endocrine and paracrine factors. All these factors are controlled by tightly regulated expression and interaction of a multitude of genes in different compartments of the ovary [16]. As one of the major classes of gene regulators, miRNAs are considered to be involved in the regulation of ovarian genes [36,82]. Several studies expanding from identification and
expression profiling to functional involvement of miRNAs in the ovary have been carried out in different animal species. Four attempts have led to identify the distinct and major population of miRNAs in 2 weeks old and adult mouse ovary [83], adult mouse ovary and testis [66], adult bovine ovary [36] and new born mouse ovary [2] through small RNA library construction and sequencing. Regardless of species these studies showed that let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145 and miR-199b to be most commonly miRNAs found in the ovary. The presence of miRNAs and their differential expression can give the primary clue for their potential role in ovarian function. However, further functional characterization of these miRNAs in different cell types of ovary (oocyte, granulosa, theca cells and ovarian stroma) at different follicular stage or at different estrus cycle remains to be elucidated. Although bioinformatic prediction and analysis of ovary specific mRNAs targets for these enriched miRNAs revealed several molecular and cellular pathways and physiological functions important for ovarian follicular development [36], atresia, ovulation as well as ovarian dysfunction, the identification of functional target mRNAs remains to be validated by appropriate wet lab experiment.

Figure 2. Gene silencing by siRNAs.
Several studies highlighted the expression and regulation of some individual miRNAs in different ovarian cells especially in oocyte and granulosa cells. After disclosing the absence or less role of sperm born miRNAs in mammalian fertilization [5], further studies were directed towards these two cell types (oocyte and granulosa). For example, the first attempt was made in 2006 and the study identified small number of miRNAs as well as some other small noncoding RNAs (rasiRNAs, gsRNAs) in mouse oocyte [105]. However, further identification of miRNAs in oocytes through direct cloning method is still missing rather more initiative has been taken for microarray or RT-PCR based miRNAs detection through homologous or heterologous approach. For example, the differential expression of microRNAs has been identified during bovine oocyte maturation and preimplantation embryo development in vitro using the homologous approach [97].

The Microarray experiments show that Dicer1 is highly expressed and functionally important in the oocytes during folliculogenesis as well as in the mature oocytes [23,69,92]. Conditional knockout of Dicer1 in growing oocytes revealed unaffected oocyte growth and folliculogenesis during the early stage but meiosis I has been found to be arrested with defective spindle organization in oocytes lacking Dicer1 [69]. Moreover, transcriptional analysis through microarray experiments has identified the major portion of the transcripts as misregulated in Dicer-deficient oocytes. These efforts not only provide initial evidence for the role of miRNAs in the oocyte but also suggested that a large proportion of the maternal genes are directly or indirectly under the control of miRNAs [69,95]. However, Suh et al. [93] studied the effect of deletion of another miRNAs processing molecules called Dgcr8 and revealed contrasting conclusion that the effects on the phenotypes in Dicer deficient oocytes are rather due to endogenous siRNAs [94]. Moreover, the expression level of miRNAs in Dgcr8 deficient oocyte found to be reduced as similar to the Dicer deficient oocyte. In addition, there was no effect due to deletion of Dgcr8 allele even from maternal and zygotic genome on the phenotype as well as mRNA profile which were very unlikely for Dicer deficient oocytes. These findings show that miRNA function is globally suppressed during oocyte maturation and preimplantation development.

However, the progress of the study in this regard is higher in case of granulosa cells compared to oocyte. For example, study of expression of miRNAs by Fiedler et al. [27] in mouse mural granulosa cells collected before and after an ovulatory dose of hCG identified miR-132 and miR-212 as highly upregulated following LH/hCG induction. Further analysis of these two miRNAs in cultured granulosa cells revealed the roles in the post-transcriptional regulation of CtBP1 gene which is known to be interacting with steroidogenic factor-1 and acts as a co-repressor of nuclear receptor target genes. Recently, it has been studied to know the role of miRNAs in human granulosa cells (GC) by transfecting 187 individual synthetic miRNA precursors that mimic endogenous precursor miRNAs representing the majority of human miRNAs [88]. Interestingly, they have screened 80 miRNAs which control both proliferation and apoptosis in ovarian granulosa cells, as well as they identified miRNAs which promote and suppress these processes utilizing a genome-wide miRNA screen. Transfection of cultured human granulosa cells with 11 out of 80 tested miRNA constructs resulted in significant increase in percentage of cells containing PCNA a cell proliferation marker. These were mir-108, mir-7, mir-9, mir-105, mir-128, mir-132, mir-141, mir-142, mir-152, mir-188 and mir-191. Eleven of the 80 miRNAs tested in the same experiment (mir-15a, mir-96, mir-92, mir-124, mir-18, mir-29a, mir-125a, mir-136, mir-147, mir-183 and mir-32) found to be promoted up to two fold accumulation of Bax - proapoptotic marker in human primary granulosa cells. However, the detailed regulatory mechanism for regulating such two processes through targeting which miRNAs by the individual miRNAs are unknown and remains to be disclosed in future investigation.

The most recent work highlighted one miRNAs (miR-224) in detail for regulation of granulosa cell proliferation and thereafter has shown to affect ovarian estrogen release in mouse [113]. In that experiment miR-224 expression was found to be regulated by TGF-â/Smads pathway through inhibiting TGF-â superfamily type I receptors (SB431542) which leads to block phosphorylation of the downstream effectors Smad2/3 in vitro in granulosa cells. The ectopic expression of miR-224 was suggested to enhance TGF- â 1-induced granulosa cell proliferation through targeting Smad4. This was a good demonstration for the notion that miRNAs could control or promote TGF- â 1-induced GC proliferation and ovarian estrogen release. However, there are many more miRNAs and their mechanism involved in the function of granulosa cells is still remaining to be elucidated. So, to further clarify the role of miRNAs in oogenesis and folliculogenesis, generation of knockouts or knocking down the individual miRNAs could help to understand their critical roles in ovarian development as well as ovarian cellular functions. Information on the regulatory role of miRNAs in the ovarian cells of ruminants compared to human and mouse are so limited and these are the open field for the researcher working on ruminant reproductive biology. Currently, the expression and functional evidence of miRNAs in the follicular theca cells in any physiological states of any species remains to be elucidated.
V. EXPRESSION AND REGULATION OF miRNAs IN THE TESTICULAR CELLS AND THEIR FUNCTIONS

MiRNAs were first detected from the testis during establishing the techniques reliable for genome-wide miRNA profiling [10,57]. A number of miRNAs differentially expressed during testicular development and bioinformatic identification of several possible male germ cell target mRNAs has been reported [116]. Further analysis revealed mir-122a targeting transition protein 2 (Tnp2) mRNA, a testis-specific and post-transcriptionally regulated mRNA in postmeiotic germ cells first suggested the miRNAs mediated posttranscriptional regulation in the mammalian testis. Small RNAs cDNA library constructed and identified 52 distinct miRNAs as well as other small noncoding RNAs (rasiRNAs and gsRNAs) in the testis [105]. The evidence for the potential involvement of the miRNA pathway in the regulation of male germ cell (GC) development were reported by localizing testis-expressed miRNAs (miR-21, let-7a, miR-122a), in the chromatoid body of male GCs and expected to have control in post-meiotic GC differentiation [48]. In 2007 Novotny and his coworkers lay out the potential involvement of miRNAs in post-transcriptional regulation in the testis by the miR-17-92 cluster during meiotic recombination [70]. In the same year several individual efforts were made to clone miRNAs from the testis in a large scale. Through small RNA cloning method Ro et al. [82] identified 141 miRNAs from the mouse testis including 29 novel miRNAs and from the pattern of expression they have suggested twenty eight candidate miRNAs which are preferentially (22) or exclusively (6) expressed in the mouse testis for further functional studies. Comparison of miRNAs pattern between immature and mature mouse testes through miRNA microarray (with 892 miRNA probes) identified 19 significantly different miRNAs expression [111]. Future studies ablating specific miRNAs using transgenic technologies or by other suitable approach will help us better understand the role of individual miRNAs in gonadal development and functionality. The expression patterns of several members of the miRNA pathway in the testis namely Dicer (Dcr), Drosha, Ago1, Ago2, Ago3 and Ago4 are identified to express in pachytene spermatocytes, round and elongated spermatids and Sertoli cells [32]. Moreover, miRNAs were found to be localized XY body of spermatocytes including the nucleolus of Sertoli cells [61]. The transgenic male mouse lacking Dcr in germ cells were found to be subfertile both due to the defect in the transition from round to elongating spermatids and production of sperm with abnormal motility [60]. Recent study has identified that about 86% of X-linked miRNAs actually escape meiotic sex chromosome inactivation (MSCI) during spermatogenesis and transcriptional silencing of genes on X & Y chromosomes was found to occurs in mid-to-late pachytene spermatocytes [89]. Furthermore, selective ablation of Dcr in Sertoli cells has led to infertility due to complete absence of spermatozoa and progressive testicular degeneration [75]. In the same study altered expression of several key genes such as Gdnf, Kitl, Man2a2, and Serpina5 which are essential for spermatogenesis, was revealed as a result of the miRNA mediated post-transcriptional control in the Sertoli cells leading to abnormal spermatogenesis. The existence, preferential and temporal differential expression of miRNAs and the involvement of their machinery genes especially Dcr in the mature and immature testis as well as in different testicular cells has evidenced the functional role of miRNAs in the physiology of testis. Despite various studies carried out on comparative expression analysis of hundreds of testicular miRNAs, there is a tremendous research gap in the investigation of exact functional role of specific miRNAs in the development and proliferation of germ cells in testis.

VI. miRNAs REGULATION OF EMBRYONIC DEVELOPMENT PROCESS AND STEM CELLS MAINTENANCE

The well-orchestrated expression of genes that are derived from the maternal and/or embryonic genome is required for the onset and maintenance of distinct morphological changes during the embryonic development. Optimum regulation of genes or critical gene regulatory event in favor of early embryonic development have been shown directly (individual miRNAs study) or indirectly (disrupting miRNAs biogenesis) under the control of miRNAs. Disruption of Dicer1 - an enzyme important for biogenesis of miRNAs and RNA interference related pathways in mammals was first demonstrated and shown that loss of Dicer1 lead to lethality early in development where Dicer1-null embryos were found to be depleted of stem cells in mouse [15]. Another report has been published in the same year to show the importance of Dicer1 in vertebrate development through inactivation of the Dicer1 gene in zebrafish and subsequently observed the early developmental arrest [107]. Shortly after, the defective generation of microRNAs has been noticed in Dicer-null mouse embryonic stem cells with severe defects in differentiation both in vitro and in vivo and re-expression of Dicer in the knockout cells has been found to rescue these defective phenotypes [44]. Additionally,
maternal miRNAs have been shown to be essential for the earliest stages of mouse embryonic development through the loss of maternal inheritance of miRNAs following specific deletion of Dicer from growing oocytes [95]. So, these initial reports suggested that miRNAs are essential for embryonic development as the effect of loss of Dicer1 could primarily arise from an inability to process endogenous miRNAs and later on to be functioning in gene regulation. While critical roles for miRNAs biogenesis in the early embryonic development well established, roles for individual miRNAs have only recently been investigated mostly in the mouse.

The role of miRNAs has been suggested first for differentiation or maintenance of tissue identity during early embryonic development in zebrafish [106]. Several attempts were made to clone miRNAs from the embryo or embryonic tissues to further understand the miRNA-mediated regulation of embryonic development. A significant number of miRNAs has been identified at specific stages of mouse embryonic development through massively parallel signature sequencing technology [65] and in bovine embryo through small RNAs library construction [25]. The coexistence of dynamic synthesis and degradation of miRNAs has been shown but overall quantity and stage-dependent miRNAs increases as the embryos develop during mouse preimplantation stage embryonic development [112]. Even, during the preimplantation stage miRNAs are shown to participate in directing the highly regulated spatiotemporally expressed genetic network as well. In vitro gain- and loss-of-function experiments showed that the expression of cyclooxygenase-2, a gene critical for implantation, is post-transcriptionally regulated by two miRNAs mmu-miR-101a and mmu-miR-199a* [20]. Another study has identified higher expression of miR-21 in the subluminal stromal cells at implantation sites on day 5 of pregnancy but not detected during pseudo-pregnancy or even under delayed implantation [39]. This revealed that the expression of mmu-miR-21 in the implantation sites regulated by the active blastocysts. Moreover, in the same study, the role of miR-21 in embryo implantation has been suggested due to targeted regulation of the Reck gene [39]. Recent microarray based miRNAs expression profiling in elongated cloned and in vitro-fertilized bovine embryos has suggested that the reprogramming of miRNAs occurred in cloned bovine elongated embryos [19]. However, status of reprogramming error in the extra embryonic tissues (or placenta) has not yet been separated which could be the main reason for the cloned pregnancy loss during the first trimester.

Recent studies identified a unique set of miRNAs expressed and its functional importance in embryonic stem cells (ES cells). Initial effort has identified that miR-290 through miR-295 (miR-290 cluster) are ES cell-specific and there after suggested that they could potentially participate in early embryonic processes such as the maintenance of pluripotency in mouse [38]. Similar study in human has also identified some clustered miRNAs (miR-296, miR-301 and miR-302: homologous to the miRNAs reported by Houbaviy et al. in mouse) specifically expressed in human ES cells and not in differentiated embryonic cells or adult tissues [93]. These clustered miRNA organization is presumably effective for coordinated regulation of their expression and regulation of common targets because a common seed is shared between some miR-290 cluster miRNAs, miR-302a-d and miR-93 [37,38]. The role of miR-290 cluster in embryogenesis has been evidenced in a study, in which the generation of a mouse mutant with a homozygous deletion of the miR-290 cluster resulted in the death of embryos [7]. By the loss- or gain-of-function studies of Dicer, DGCR8 and ES-related miRNA genes such as miR-290-295 cluster have strongly suggested that miRNAs play an important role in ES cell maintenance, differentiation [12,87] and lineage determination [41,44,96,102]. Despite the fact that knowledge on the role of microRNAs in the embryonic development and stem cell maintenance, differentiation and lineage in mouse and human is increasingly building, it is yet to be elucidated for ruminants.

VII. miRNAs IN THE REGULATION OF EPIGENETIC PROCESSES

The term epigenetics refers to all heritable changes in gene expression that are not associated with concomitant alterations in the DNA sequence. Reversible DNA methylation and histone modifications are known to have profound effects on controlling gene expression. Correct DNA methylation patterns are paramount for the generation of functional gametes with pluripotency states, embryo development, placental function and the maintenance of genome architecture and expression in somatic cells. Aberrancies in both the epigenetic and the miRNA regulation of genes have been documented to be important in diseases and early development. Interestingly, it has been evident that there is an effect of miRNAs on epigenetic machinery. On the other hand miRNA expression also found to be controlled by epigenetic mechanisms. Very little is known about the miRNAs mediated epigenetic process or epigenetic control of miRNAs expression, which are potentially involved in regulating reproduction and early development. It has been shown that Dicer could play a role in heterochromatin formation [29]. In addition, Dicer-deficient mutants are
shown to reduce epigenetic silencing of expression from centromeric repeat sequences as a result of alterations in DNA methylation and histone modifications [44]. As contradictory to this, it was also observed with no apparent changes in the centromeric heterochromatin later on [68]. However, recent studies by the loss- or gain-of-function studies such controversial results has become clear, where the Dicer deficient stem cells were found to have reduced levels of both de novo DNA methylation and DNA methyltransferases (Dnmts) [12,87] as well as increased telomere recombination and elongation [12]. These results supported a model in which the miR-290 cluster maintains ES cells by controlling de novo DNA methylation via Rbl2 and indirectly telomere homeostasis and by repressing the self-renewal program through modulating the epigenetic status of pluripotency genes upon differentiation [reviewed in 101].

Recently, aberrant epigenetic reprogramming of imprinted miR-127 in cloned murine embryos has been reported and found a correlation with the aberrant epigenetic reprogramming of the mouse retrotransposon-like gene Rtl1 [26]. MicroRNA-mediated switching of chromatin remodeling complexes in neural development by repression of BAF53a have observed in mouse [115]. Where, the repression is accomplished through the 3’ UTR of BAF53a and mediated by the simultaneous activities of miR-9* and miR-124. Repressor-element-1-silencing transcription factor participates in this switch by repressing miR-9* and miR-124, thereby permitting BAF53a expression in neural progenitors. Interestingly, it has been reported that the aberrant DNA methylation and histone modifications could simultaneously induce silencing of miRNAs in colorectal cancer [9]. The relation of miRNA and epigenetics is presently being elucidated. So, much less is known about the specific miRNA and their targets to regulate epigenetic machinery or epigenetic regulation of specific miRNAs that are required for normal physiological condition or for any phenotypic effects, but this area of research is rapidly moving forward.

VIII. IMPLICATION OF sncRNAs FOR RUMINANT REPRODUCTIVE BIOLOGY AND CHALLENGES

Non-coding RNAs comprise the majority of the mammalian transcriptome and have been suggested to play an important role in the regulation of gene expression. They are important in most epigenetic mechanisms as is exemplified by the role of small RNAs in silencing of transposable elements, microRNAs in gene expression control, large RNAs in X-chromosome inactivation and DNA imprinting and “heritable” RNAs in non-mendelian epigenetic inheritance. Moreover, DNA methylation and histone modifications can be directed by different types of ncRNAs. Among the sncRNAs, miRNAs seem well suited to maintain the delicate balance between normal reproductive biology, system development and tissue maintenance versus deregulated growth and tumor formation. These small non-coding RNAs have been found to play a central role in various cellular activities, including developmental processes, cell growth, differentiation and apoptosis, cell–cell communication, inflammatory and immune responses through gene expression stability. As many of these processes are an integrated part of gonadal functions, germ cell formation-development-differentiation, uterine and oviductal cellular activities during different stage of reproduction and steroid synthesis, it is possible to postulate the potential role of miRNAs in regulator of reproductive biology along with other physiological functions. Alteration of the expression of miRNAs in any of these processes could lead to subsequent infertility, reproductive and other steroid-dependent disorders with ultimate failure in reproduction. Emphasis is placed on the necessity to enhance our understanding of the miRNAs mediated regulation of cellular process as after initial observation of defective morphology, development, fertility and abnormal cellular development in Dicer deficient mouse.

Being an important gene regulator, miRNAs could be an interesting avenue to resolve lot of questions on different regulatory mechanisms of ruminant’s reproductive process. Posttranscriptional gene regulation by the miRNAs during different periods of ovarian follicular development, atresia and luteolysis could be an interesting field of investigation in ruminants. Since the ovarian follicle is a complex structure composed of different types of cells that are functionally related and constantly changing and differentiating. Experimentation is required but remains to be elucidated for the role of miRNAs in the interaction between granulosa and theca cells which is essential for estrogen biosynthesis process. In vitro culture models for a single cell type (primary granulosa), co-cultures of theca and granulosa cells or whole follicle cultures could be utilized for elucidating such miRNAs mediated regulation to overcome the technical difficulties in in-vivo experiment. The first and foremost requirement to get in of this, the whole set of miRNAs in different ovarian cells should be indentified in ruminants. To accomplish this, miRNAs microarray could be a useful approach either by using arrays from ruminants or by heterologous array from mouse or human along with

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direct identification through construction of ovarian cell specific small RNAs library. In addition to identification, miRNA microarray could be also useful to describe dynamic changes in miRNA transcript levels in closely related to regulatory events of gene expression for successful follicular development to explain how this is all managed by the different ovarian cell types and its interactions with environmental conditions. Until recently, whatever the mRNA transcripts discovered specific to oocyte and granulosa cells in mouse or human and important for fertility could be utilized and checked for their altered expression interceded by miRNAs. Although hundreds of genes, which are important for ovarian physiology, are predicted to be potential target of miRNAs [36], but all these targets should be validated to elucidate key points of such regulation. Thereafter, it might be possible to draw a fine description of the role of miRNAs in the molecular mechanisms of the dynamic processes occurring in these different compartments of ovary during follicular development and might provide insight into how we might be able to enhance reproductive efficiencies.

In the absence of transcription, synthesis of hundreds of new products and disappearance of many proteins during oocyte maturation after germinal vesicle breakdown and early embryogenesis indicating fine regulation of hundreds of transcripts by a mechanism other than transcription. These changes could possibly largely rely on and controlled by miRNAs, but it is still remains to be elucidated. In addition, it has been evidenced that the bidirectional interactions between oocyte and somatic cells control folliculogenesis, where oocyte secretes soluble paracrine factors that act on its adjacent granulosa cells, which in turn regulate oocyte development in bi-directional communication axis [30]. Further experimentation is required to know the detail of such mechanism. So, the role of miRNAs in paracrine signaling and gapjuntional exchange and control of regulatory molecules through intercellular communication between oocytes and granulosa cells is another interesting source of experimentation to supplement our existing knowledge on bi-directional communication between these two types of cells.

A large number of target genes for a single miRNA and multiple miRNAs targeting the expression of one gene have been recognized as a major challenge drawback in the assessment of the role of specific miRNAs and establishing precise miRNA-target networks. Moreover, the identification of functional targets represents a major hurdle in our understanding of microRNA function for complex phenomena of reproduction in different ruminant species due to lack of complete genomic information, available bio-informatic tools and suitability to carry out in-vivo functional studies. A few number of knockout studies in mice have been carried out to show the involvement of regulatory miRNAs in mammalian reproduction. However, the knowledge on the functions of specific miRNAs from mouse knockout models cannot be systematically applied to ruminants. So, for large ruminant, the production of transgenic animals could help to elucidate miRNAs mediated regulation of reproductive process in vivo. However, the success of such approaches is limited due to technical difficulty, cost of making null miRNA transgenics and extended time frame required to observe the effect in reproductive process in ruminants.

Presently, our understanding of non-coding RNAs specially miRNAs function in reproductive biology is very limited and much remains to be uncovered in this exciting field of investigation. Better understanding of small non-coding RNAs, especially miRNA-mediated regulatory effects could be potentially used for regulation of ruminant reproductive processes including fertility and for treatment of reproductive and other steroid-dependent disorders in near future and could be directly applied to other species as model.

IX. CONCLUSION

Non-coding RNAs comprise the majority of the mammalian transcriptome and have been suggested to play an important role in the regulation of gene expression. In contrast to the uncertainty surrounding the function of most mammalian ncRNAs, imprinted macro ncRNAs have clearly been shown to regulate flanking genes epigenetically and small non-coding RNAs have been shown to have tremendous transcriptional regulation for normal physiology or disease condition of different types of tissues and cells. Among the sncRNAs, miRNAs are the well characterized one which could maintain the delicate balance between normal reproductive biology, system development and tissue maintenance versus deregulated growth and tumor formation. The studies on the role miRNAs in disease development are much extensive than on reproductive biology and furthermore very limited in ruminant species compared to human and mouse. Conditional Dicer1 knockout mice have been used to show the consequences that the lack of miRNA have on ovarian, testicular, oviductal, uterine, oocyte, and embryonic function and development. To date, much of the work on miRNAs has focused on expression profiling rather than their regulation and functional
characterization within specific tissues and cells or during the reproductive process. However, this area of research is rapidly moving forward and it is expected that a lot of information regarding miRNA-mediated posttranscriptional gene regulation and their epigenetic regulation in ruminant reproduction biology will be known within the next several years. Studies to identify the specific miRNAs, their target genes and post transcriptional regulatory network will further shed light on the importance of specific miRNA both for the development and function of reproductive tissues as well as disease condition. Once relevant miRNAs and functional targets are identified, possible clinical use for these molecules will represent the next front line and may lead to novel strategies for better enhancing or manipulating reproductive efficiency.

REFERENCES


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